Interphase phosphorylation of the Drosophila nuclear lamin: site-mapping using a monoclonal antibody

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SUMMARY

The Drosophila nuclear lamin is highly phosphorylated during interphase. Two interphase isoforms, differing in degree of phosphorylation, can be distinguished by one-dimensional SDS-polyacrylamide gel electrophoresis. One migrates with an apparent mass of 74 kDa (lamin Dm1); the other is more highly phosphorylated and migrates as a 76 kDa protein (lamin Dm2). We generated a monoclonal antibody, ADL84 which binds to lamin Dm1 but not lamin Dm2. Binding of ADL84 to lamin Dm2 was restored by phosphatase treatment of immunoblots containing lamins. Immunoprecipitation with ADL84 demonstrated that purified Drosophila nuclear lamins Dm1 and Dm2 are present as a random mixture of homo- and heterodimers. Indirect immunofluorescence experiments suggest that lamin Dm1 is present in all Drosophila cell types. The epitope for ADL84 was mapped by analyzing binding to bacterially expressed lamin deletion mutants and subsequently by screening for point mutants (randomly generated by polymerase chain reaction) which were not recognized by ADL84. The ADL84-epitope encompasses amino acids R22-PPSAGP (arginine 22-proline 28). Peptide competition experiments demonstrated directly that phosphorylation of serine 25 impedes lamin binding by ADL84. This suggests that serine 25 is the lamin Dm2-specific phosphorylation site.

Key words: lamin, nuclear envelope, phosphorylation

INTRODUCTION

Nuclear lamins are major components of the nuclear lamina, a fibrous layer of proteins underlying the inner nuclear membrane and surrounding the nucleus. In Xenopus oocytes, lamins form a network composed of 10 nm fibers which cross-over at regular intervals (Aebi et al., 1986; Whytock et al., 1990). The detailed in situ structure of lamins in other cell-types is not well-known (but see Hill and Whytock, 1993). Lamins interact with the inner nuclear membrane via an isoprenylated carboxy-terminal tail (Weber et al., 1989; Kitten and Nigg, 1991) and/or a lamin-receptor in the nuclear membrane (Worman et al., 1988, 1990; Hennekes and Nigg, 1994). In vitro, lamins also bind chromatin (Burke, 1990; Hoger et al., 1991; Yuan et al., 1991; Glass et al., 1993) and DNA of specific sequence (Ludérus et al., 1992). Results from a number of experiments suggest that lamins play a structural role in nuclear formation (Benavente and Krohne, 1986; Ulitzur et al., 1992) and perhaps in nuclear function (Newport et al., 1990; Meier et al., 1991).

Multiple posttranslational modifications occur on lamins (Nigg, 1992). During mitosis, phosphorylation of one or a few specific lamin sites correlates with lamina disassembly (Smith and Fisher, 1989; Heald and McKeon, 1990; Peter et al., 1990, 1991; Ward and Kirshner, 1990; Goss et al., 1994). Rat liver lamin B was shown to be reversibly demethylated during mitosis (Chelsky et al., 1987). During interphase different stimuli can cause specific lamin phosphorylation via protein kinase C (Hornbeck et al., 1988; Tsuda and Alexander, 1990; Kasahara et al., 1991; Martell et al., 1992; Goss et al., 1994) or other kinases (Molloy and Little, 1992; Eggert et al., 1993). In one case where a biological effect of interphase phosphorylation was demonstrated, Hennekes et al. (1993) showed that phosphorylation of chicken lamin B2 at a site near its nuclear localization signal inhibits lamin import into the nucleus.

In Drosophila three lamin cDNAs, coding for two different proteins, were identified (Gruenbaum et al., 1988; Bossie and Sanders, 1993). The protein encoded by one of these cDNAs (lamin C) is developmentally regulated (D. Riemer et al., unpublished). The other two code for the major Drosophila embryo lamin, Dm0. Lamin* Dm0 has an apparent molecular mass of 76 kDa as determined by SDS-polyacrylamide gel electrophoresis (PAGE) and is rapidly processed in the cytoplasm into a form migrating at 74 kDa (lamin Dm1). Lamin Dm1 is imported into the nucleus where about 50% is posttranslationally modified resulting in a slower migrating form (76 kDa) called lamin Dm2. In vivo pulse-chase studies

*Unless indicated otherwise, for Drosophila, the term lamin refers solely to protein products encoded by the Drosophila lamin Dm0 gene.
indicate that lamins Dm1 and Dm2 are in equilibrium. Treatment of lamins Dm1 and Dm2 with phosphatase results in a single form that comigrates with lamin Dm1 after SDS-PAGE. In conjunction with results of in vivo labeling, this suggests that lamin Dm2 arises by specific phosphorylation of lamin Dm1 (Smith et al., 1987; Smith and Fisher, 1989).

Here, we describe the isolation and characterization of a monoclonal antibody, ADL84, which is specific for Drosophila lamin Dm1 and does not bind lamin Dm2. We used this antibody to show that isolated interphase laminas occur as mixed dimers, indicating that lamins Dm1 and Dm2 interact randomly with each other. Mapping of the epitope for ADL84 shows that the lamin Dm2-specific phosphorylation takes place in the NH2-terminal ‘head’-domain of the lamin, most likely at serine 25.

**MATERIALS AND METHODS**

**Generation of monoclonal antibodies**

We employed an immunization protocol that was shown to enhance the recovery of antibodies against relatively less antigenic epitopes (Matthew and Sandrock, 1987; Vermeersch et al., 1992). At day 1 Balb/c mice were injected intraperitoneally with 150 μg of Drosophila lamin Dm0 produced in Escherichia coli (see below) mixed with an equal volume of MPL plus TDM emulsion adjuvant (RIB Immunochemical Research Inc., Hamilton, MT). After 10 minutes, 24 hours and 48 hours, mice were injected with a 2 mg/ml solution of cyclophosphamide (Sigma, St Louis, MO) in 0.9% (w/v) NaCl to a concentration of 100 mg/kg body weight. This set of four injections (one of antigen followed by three of cyclophosphamide) was repeated at day 16. At days 32, 49 and 63, mice were immunized with 150 μg each of authentic interphase Drosophila lamins Dm1 and Dm2, mixed with adjuvant. At day 66, one mouse was killed, and spleen cells were used for monoclonal antibody (mAb) generation according to standard protocols (Harlow and Lane, 1988).

Hybridomas were tested for specific antibody production using an enzyme linked immunosorbent assay (Harlow and Lane, 1988) with E. coli expressed or authentic lamin as antigen. Several anti-Drosophila lamin antibody secreting hybridomas were selected and cloned by repeated limiting dilution until greater than 90% of the resulting clones produced the specific antibody. Antibody isotype was determined by enzyme linked immunosorbent assay using isotype-specific secondary antibodies (Fisher Scientific, Pittsburgh, PA).

**Expression of the full-length Drosophila lamin and deletion mutants in E. coli**

Full-length Drosophila lamin and lamin Dm0 deletion mutants were expressed in E. coli and purified as described elsewhere (N. Stuurman et al., unpublished).

**Protein purification**

Authentic interphase lamin was purified from 6- to 18-hour-old Drosophila embryos essentially as previously described (Lin and Fisher, 1990). All steps were performed at 4°C except when indicated. Frozen, dechorionated embryos were thawed in 9 volumes (one volume equals the starting volume of embryos) of buffer A (50 mM sodium phosphate, pH 8.0, 15 mM MgCl2, 1.0 M sucrose, 0.5 mM DTT) supplemented with protease inhibitors (1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml chymostatin, 2 μg/ml pepstatin-A). Embryos were broken using a Dounce homogenizer (5 strokes, tight pestle) and the homogenate was filtered through two layers of 120 μm nylon mesh. Nuclei were collected by centrifugation for 15 minutes at 20,000 g in a swinging-bucket rotor through a cushion of buffer A supplemented with protease inhibitors. After a single wash in buffer A supplemented with protease inhibitors (by resuspension and centrifugation for 10 minutes at 8,000 g), nuclei were resuspended in one volume buffer B (10 mM sodium phosphate, pH 8.0, 5 mM MgCl2, 0.5 mM DTT, protease inhibitors). DNase I and RNase A were added to final concentrations of 10 μg/ml each and the suspension was incubated for 15 minutes at 23°C. Nuclei were recovered by centrifugation for 10 minutes at 5,000 g, resuspended in 0.9 volumes of buffer C (10 mM sodium phosphate, pH 8.0, 0.1 mM MgCl2, 250 mM sucrose plus protease inhibitors) and Triton X-100 was added to 2% (w/v) from a 20% (w/v) solution in water. After a 10 minute incubation the suspension was centrifuged for 10 minutes at 2,000 g. The pellet was resuspended in 0.5 volumes of buffer D (100 mM Tris–HCl pH 8.1 (4°C), 0.1 mM MgCl2, 250 mM sucrose, 0.1% (w/v) Triton X-100, protease inhibitors) and NaCl was added to a final concentration of 0.5 M from a 1.0 M stock solution. After centrifugation for 10 minutes at 10,000 g the supernatant was used immediately for immunofluorescence analysis of lamins.

To prepare an anti-Drosophila lamin affinity resin, antiserum was raised in rabbits. Specific IgG was affinity-purified from this serum using as the immobilized affinity ligand, bacterially expressed lamin Dm0 fragment L1-522 (containing residues 1 to 522 of full length lamin Dm0) covalently bound to glutaraldehyde-activated glass beads (Boehringer, Mannheim, FRG). Bound IgG was eluted using 100 mM glycine–HCl, pH 2.3, 500 mM NaCl, 0.1% (w/v) Triton X-100, thus selecting for anti-lamin IgG which does not bind lamin at low pH. The antibody was cross-linked to Protein-A agarose (Schleicher and Schuell, Keene, NH) using dimethylpimelimidate as described (Harlow and Lane, 1988).

The 0.5 M NaCl extract of nuclease treated, Triton X-100 treated nuclei was mixed with anti-Drosophila lamin-Protein A agarose for 4 hours at 4°C. The resin was then washed with 40 column volumes of 20 mM Tris–HCl, pH 8.1 (4°C), 0.5 M NaCl, 5 mM EDTA, 0.1% (w/v) Triton X-100, and bound lamin was eluted with 50 mM glycine–HCl, pH 2.3, 0.5 M NaCl, 0.1% (w/v) Triton X-100. Eluted material was immediately neutralized by addition of NaHPO4 to 50 mM; lamin-containing fractions were pooled and stored at −80°C.

**Epitope mapping by random mutagenesis**

For random mutagenesis by polymerase chain reaction (PCR) (Zhou et al., 1991), pET-DmLFL (N. Stuurman et al., unpublished) was linearized by digestion with EcoRI and used at a final concentration of 50 ng/ml in a PCR reaction containing 10 mM Tris–HCl, pH 9.0 (25°C), 50 mM KCl, 0.1% (w/v) Triton X-100, 1.5 mM MgCl2, 0.2 mM of each dNTP, 22 units/ml Taq DNA polymerase (Boehringer, Mannheim, FRG), 10% (v/v) dimethylsulfoxide, 0.5 pM T7 primer, 0.5 pM primer 5’-TCTTGCAGTCGTGG. The reaction was performed in multiple 100 μl-aliquots with the following temperature profile: 5 minutes at 94°C followed by 30 cycles (1 minute and 20 seconds at 94°C, 1 minute at 45°C, 4 minutes at 72°C) and a final extension for 10 minutes at 72°C. The product was phenol/chloroform extracted, ethanol precipitated, digested with NdeI and HindIII, and gel-purified. This fragment was ligated into pET-22b (Novagen, Madison, WI) which had been digested with NdeI and HindIII and had been dephosphorylated. The ligation mixture was electrotansformed into HMS174(DE3)pLysS (Novagen). Colonies were duplicated onto nitrocellulose membranes and induced to produce recombinant protein by placing the filter on an agar plate containing 1 mM IPTG. After three hours at 37°C, the filter was removed and cells were lysed by freezing (5 minutes at −80°C) and thawing (5 minutes at 37°C) twice. Lysed cells were labeled in situ using ADL84 followed by anti-mouse IgG conjugated to alkaline phosphatase (Kierkegaard and Perry Laboratories Inc., Gaithersburg, MD) and a one-solution phosphatase substrate (Kierkegaard and Perry Laboratories Inc.). The same filter was subsequently labeled with affinity-purified polyclonal anti-Drosophila lamin IgG followed by anti-rabbit IgG conjugated to hors eradish peroxidase. Detection was with an enhanced chemiluminescence system (ECL from Amersham, Arlington Heights, IL).
Alkaline phosphatase-conjugated anti-mouse IgG does not bind rabbit IgG; horseradish peroxidase-conjugated anti-rabbit IgG does not react with mouse IgG (not shown). Colonies which reacted with the polyclonal anti-lamin antibody but not with ADL84 were selected. Plasmids were isolated from the colonies, transformed into XL-1 Blue and sequenced using ssDNA (released with helper phage) and the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH).

**SDS-PAGE and immunoblotting**

Proteins were separated by SDS-PAGE according to the method of Laemmli (1970) and transferred to nitrocellulose using the method described by Towbin et al. (1979). Blots were stained with a 0.2% (w/v) solution of Ponceau-S (Sigma) and destained with 12 mM HCl to visualize transferred proteins. Blots were equilibrated in PBS containing 0.5% (v/v) Tween-20 and incubated with primary antibody (affinity-purified polyclonal rabbit anti-\textit{Drosophila} lamin IgG at 50 ng/ml, or culture supernatants from hybridomas secreting anti-\textit{Drosophila} lamin antibodies at a 10- to 100-fold dilution) for 2-16 hours at room temperature. Bound antibodies were detected using goat anti-rabbit or goat anti-mouse IgG conjugated to alkaline phosphatase (Kierkegaard and Perry Laboratories Inc., Gaithersburg, MD) and a one-solution phosphatase substrate (Kierkegaard and Perry Laboratories Inc.).

**In situ alkaline phosphatase treatment of immunoblots**

Lamins Dm$_1$ and Dm$_2$ were separated by SDS-PAGE and blotted onto nitrocellulose strips. These strips were incubated for 1 hour at room temperature with 10 mg/ml BSA in PBS containing 0.5% (v/v) Tween-20. They were washed three times with 50 mM glycine, pH 9.5, 0.1% (w/v) Tween-20 and incubated for 90 minutes at 37°C with 500 U/ml calf intestine alkaline phosphatase (Boehringer, Mannheim, Germany). Controls were incubated similarly but without added enzyme. Immunoblot strips were incubated with antibodies essentially as described above; detection of immunoreactivity was with goat anti-mouse IgG conjugated to horseradish peroxidase and a one-solution peroxidase substrate (Kierkegaard and Perry Laboratories Inc.).

**Immunoprecipitation**

For immunoprecipitation monoclonal antibodies were bound to Protein-G Sepharose (Pharmacia, Piscataway, NJ) by incubating 150 μl of culture supernatant of antibody secreting hybridomas supplemented with 100 mM Tris-HCl, pH 8.0, with 50 μl Protein-G Sepharose for two hours at room temperature. The beads were washed three times with 10 mM sodium phosphate (pH 8.0), 0.5 M NaCl, 5 mM EDTA, 0.1% (w/v) Triton X-100; 15 μl of beads were incubated for 16 hours at room temperature with 30 μl of affinity-purified authentic \textit{Drosophila} lamin Dm$_1$ and Dm$_2$ diluted to 2 μg/ml in the same buffer. The Sepharose beads were washed five times with the same buffer and boiled in SDS sample buffer.

**Immunofluorescence**

Indirect immunofluorescence microscopy on third instar larval salivary gland squash preparations was performed as described previously (Fisher et al., 1982).

**Peptide competition assays**

Peptides were tested in an antibody capture assay with antigen competition (Harlow and Lane, 1988). Peptides L18-32 (T$^{18}$STPRPP-PVTPQVPLRPMT) were synthesized and HPLC purified to about 80% purity by Neosystem Laboratoire (Strasbourg, France). Microtiter plates were coated with lamin Dm$_0$ (0.7 pmol per well) and blocked with BSA. Affinity-purified ADL84 (3.1 nM) in PBS containing 0.1% Tween-20 was preincubated for 75 minutes at 37°C with peptides at the concentrations indicated in Fig. 7. Subsequently, 100 μl of the antibody/peptide mixture was added to each well and incubated for a further 30 minutes at 37°C. After washing with PBS containing 0.1% Tween-20, bound antibody was detected using horseradish peroxidase-conjugated swine anti-mouse Ig (Dako, Rostrup, Denmark) and o-phenylenediamine as a substrate. The reaction product was quantified by measuring the absorbance at 495 nm.

**RESULTS**

**ADL84, a monoclonal antibody specific for lamin Dm$_1$**

To study posttranslational modification of the \textit{Drosophila} nuclear lamin, we generated monoclonal antibodies directed against this protein. When immunoblots containing both interphase isoforms (lamins Dm$_1$ and Dm$_2$) were probed with these antibodies, one (ADL84) apparently recognized lamin Dm$_1$ but not lamin Dm$_2$ (Fig. 1, lane 1). ADL84 also recognized full-length \textit{Drosophila} lamin expressed in bacteria (not shown). Another monoclonal antibody (ADL67) bound both interphase isoforms (lamins Dm$_1$ and Dm$_2$) with apparently similar affinity (Fig.1, lane 3). Five other mAbs reacted with both lamins Dm$_1$ and Dm$_2$ (not shown).

Previously, it was shown that lamin Dm$_2$ arises by specific phosphorylation of lamin Dm$_1$, most likely on one or more serine residues (Smith et al., 1987). To test whether phosphorylation of lamin Dm$_2$ inhibits binding of ADL84, we treated lamins Dm$_1$ and Dm$_2$ separated by SDS-PAGE and immobilized on immunoblots, with alkaline phosphatase before incubation with mAbs. As shown in Fig. 1, lane 2, phosphatase treatment of lamin Dm$_2$ fixed on nitrocellulose fully restored its reactivity with ADL84. Apparently, the lamin Dm$_2$-specific phosphorylation decreases substantially the affinity of ADL84 for the \textit{Drosophila} lamin. This suggests that the lamin Dm$_2$-specific phosphorylation site colocalizes with the epitope for ADL84. Alternatively, specific phosphorylation of lamin Dm$_2$ to generate lamin Dm$_2$ causes a structural change in the epitope for ADL84 thus interfering with binding of this antibody; this change would have to survive SDS-PAGE and immunoblot analysis.

**Isolated interphase lamins Dm$_1$ and Dm$_2$ exist as a random population of mixed dimers**

Lamins, like other intermediate filament proteins, form a two-chain (dimeric) coiled-coil by the parallel alignment of

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**Fig. 1.** A monoclonal antibody specific for one of the two \textit{Drosophila} interphase lamin isoforms. Authentic \textit{Drosophila} lamins affinity-purified from 6- to 18-hour-old embryos were separated on an SDS-8% polyacrylamide gel and blotted to nitrocellulose. Separate blot strips were incubated with (lanes 2 and 4) or without (lanes 1 and 3) alkaline phosphatase, followed by ADL84 or ADL67 as indicated. Bound mAbs were detected using a colorimetric assay system. Migration positions of lamin Dm$_2$ (76 kDa) and lamin Dm$_1$ (74 kDa) are indicated on the left-hand side of the figure.
molecules (Aebi et al., 1986; Parry et al., 1986). This raised the question of whether the two interphase isoforms, lamins Dm1 and Dm2, form obligate homodimers, obligate heterodimers, or show no preference for themselves or each other. We addressed this question using ADL84 to immunoprecipitate selectively lamin Dm1 under conditions where dimer formation takes place but no higher order polymerization is evident (Lin and Fisher, 1990). If the lamin isoforms formed obligate homodimers, only lamin Dm1 would be found in the fraction bound to ADL84. In the case of obligate heterodimers, lamins Dm1 and Dm2 would be found in equimolar amounts in the bound fraction. If the lamin isoforms showed no preference for themselves or each other, one would expect one molecule of lamin Dm2 for two molecules of lamin Dm1 (a lamin Dm2 to Dm1 ratio of 0.50) in the ADL84 bound fraction.

Affinity-purified interphase lamins were diluted in 10 mM phosphate buffer, pH 8.0 containing 0.5 M NaCl, 5 mM EDTA and 0.1% (w/v) Triton X-100 (a condition which allows dimer formation but prevents higher-order polymerization) and mixed with Protein G-Sepharose beads which had been preadsorbed with either ADL84 or ADL67. ADL67 apparently reacts with both interphase lamin isoforms equally (see Fig. 1). It is evident from Fig. 2 (lane 84, UB) that lamin Dm1 was efficiently depleted from the unbound fraction by immunoprecipitation with ADL84, while a substantial amount of lamin Dm2 remained. Comparison with the unbound fraction after immunoprecipitation with ADL67 (which binds both lamin isoforms) demonstrated the specificity of ADL84 for lamin Dm1 under the conditions used for immunoprecipitation. The fraction bound to ADL84 was enriched in lamin Dm1 but contained a substantial amount of lamin Dm2 remained. Comparison with the unbound fraction after immunoprecipitation with ADL67 which binds both lamin isoforms) demonstrated the specificity of ADL84 for lamin Dm1 under the conditions used for immunoprecipitation. The fraction bound to ADL84 was enriched in lamin Dm1 but contained a substantial amount of lamin Dm2 (Fig. 2, lane 84, B). Densitometry revealed that the ratio of lamins Dm2:Dm1 (normalized to the ratio of these isoforms in the starting material) was 0.53±0.07 (n=4). The ratio of lamins Dm2:Dm1 bound to mAb ADL67 (normalized to

Fig. 2. Immunoprecipitation of isolated authentic Drosophila lamins with ADL84. Isolated lamins were immunoprecipitated with the lamin Dm1-specific mAb ADL84, or with ADL67 which recognizes both lamins Dm1 and Dm2. Immunoprecipitation was carried out under conditions that allow lamins to form dimers (see text). Shown is an immunoblot probed with affinity-purified polyclonal anti-Drosophila lamin antibodies which recognize both lamins Dm1 and Dm2 equally. Lane T, total before immunoprecipitation; lanes UB, unbound material after immunoprecipitation with the indicated mAb; lanes B, material bound after immunoprecipitation with the indicated mAb. Equivalent amounts of total, bound and unbound material were loaded. Migration positions of lamins Dm1 and Dm2 are indicated on the right-hand side of the figure.

Fig. 3. Indirect immunofluorescence localization of ADL84 reactivity in salivary gland cells. Squashed salivary glands were fixed and incubated with culture supernatant from ADL84 (A and B) or culture supernatant from a hybridoma secreting an unrelated Ig (C and D). Bound antibody was detected with rhodamine conjugated anti-mouse IgG. Phase contrast (A and C) and fluorescence micrographs (B and D) are shown. Bar in D, 20 μm (applies to all panels).
the ratio of the two forms in the starting material) was 0.96±0.03 (n=4). Evidently, isolated lamins Dm1 and Dm2 do not show any preference for themselves or each other at the level of dimer formation. Moreover, ADL84 and ADL67 react with non-denatured lamins in solution as well as with SDS-denatured lamins on immunoblots.

**Indirect immunofluorescence analysis of *Drosophila* cells and tissues with ADL84**

Tissue culture supernatant of ADL84 was used for in situ localization. Results (Fig. 3) show that ADL84 binds to the nuclear periphery of *Drosophila* salivary gland nuclei, suggesting that the antibody also recognizes lamin Dm1 in situ. Previously, it was shown that most, if not all, cell nuclei of *Drosophila melanogaster* bind a polyclonal antibody specific for lamins Dm1 and Dm2 (Whalen et al., 1991). To determine if any cells or tissues lacked lamin Dm1, ADL84 was used to label cryosections through all stages of the *Drosophila* life cycle. All detectable nuclei were labeled with ADL84 (not shown). To exclude in vitro phosphatase activity, stained cryosections of embryos were analyzed by immunoblotting. No alteration in the ratio of lamins Dm1 and Dm2 was seen (not shown) indicating that the observed staining pattern was not caused by an in vitro conversion of lamin Dm2 into Dm1. These data suggest that all individual nuclei contain a significant amount of lamin Dm1.

**Mapping the ADL84 epitope by random mutagenesis**

To gain insight into the location of the lamin Dm2-specific phosphorylation site, we mapped the epitope for ADL84. First, we tested binding of ADL84 to two deletion mutants of the *Drosophila* lamin expressed in *E. coli* (schematically represented in Fig. 4A). ADL84 recognized a fragment formed by the first 179 NH2-terminal amino acids of *Drosophila* lamin Dm0 (Fig. 4B, lanes 3). ADL84 did not bind to the fragment designated Headless which consists of all but the first 56 NH2-terminal lamin amino acids (Fig. 4B, lanes 2). Evidently, the epitope for ADL84 is located in the first 179 amino acids of the molecule, and is most likely within the NH2-terminal 56 amino acids.

To map the ADL84 epitope more precisely, random mutations were PCR-generated in the coding region for DmL-179. The mutated fragments were ligated into pET-22b and transformed into a host which expressed the T7 lysozyme gene (HMS174(DE3)pLysS). We chose a host containing the T7 lysozyme gene to ensure cell lysis by simple freezing and thawing. Colonies were transferred to nitrocellulose filters and expression of the mutant protein was induced by transfer of the filters to plates containing IPTG. After induction, cells were lysed and colonies were selected which could be labeled in situ with affinity-purified rabbit anti-lamin antibodies (Fig. 5, lower panel) but not with ADL84 (Fig. 5, upper panel). Of about 2,000 colonies analyzed, 12 satisfied these criteria. Cells from all 12 colonies were grown separately in suspension culture, induced to express the mutant protein with IPTG and total cell lysates were analyzed by immunoblotting using ADL84 (Fig. 6 upper panel) and affinity-purified polyclonal anti-lamin antibody (Fig. 6, lower panel). All showed a highly reduced reactivity of the mutant protein towards ADL84 but maintained full reactivity with the polyclonal anti-lamin antibody. Two representative examples are shown (Fig. 6, lanes 1 and 2).

Sequencing of these 12 clones revealed that all were mutated in an area coding for amino acids 22-28. All mutations resulted in an amino acid change (Table 1). Three mutants were sequenced throughout their coding sequence and were found not to have any additional mutations. These data indicate that
the epitope for ADL84 is formed by amino acid sequence R\textsuperscript{22}PPSAGP. The location of a serine in the middle of this sequence suggests that this serine is the site specifically phosphorylated in lamin Dm\textsubscript{2}.

Peptide competition experiments demonstrate that phosphorylation of serine 25 inhibits binding to ADL84

To corroborate conclusions drawn from epitope mapping by random mutagenesis, we synthesized the peptide representing amino acids 18-32 of lamin Dm\textsubscript{0} (L18-32) and tested its ability to bind to ADL84 in a competition assay. As shown in Fig. 7, this peptide inhibits binding of ADL84 to lamin Dm\textsubscript{0} immobilized on microtiter plates; 50\% inhibition is observed at about a 13-fold molar excess of peptide over antibody. An unrelated control peptide did not inhibit binding of ADL84 to lamin Dm\textsubscript{0} (Fig. 7). Similarly, L18-32 did not inhibit binding of another anti-	extit{Drosophila} lamin mAb to lamin Dm\textsubscript{0} (not shown).

To determine whether phosphorylation of serine 25 influenced binding of ADL84 we also tested a variant of the peptide L18-32 that was chemically phosphorylated at the serine residue corresponding to serine 25 in the full length lamin. This phosphorylated peptide inhibited binding of ADL84 to immobilized lamin Dm\textsubscript{0} with an efficiency that was about 19-fold lower than the unphosphorylated peptide (Fig. 7); 50\% inhibition occurred at a 250-fold molar excess of the phosphorylated peptide over antibody. Alkaline phosphatase treatment of the chemically phosphorylated peptide increased its ability to compete for binding to ADL84 to levels comparable to the original, unphosphorylated peptide (50\% inhibition at an 18-fold molar excess of peptide over antibody). Thus, we conclude that the epitope for ADL84 is contained within amino acids 18-32 of lamin Dm\textsubscript{0} and that phosphorylation of serine 25 inhibits lamin binding to ADL84.

**DISCUSSION**

Interphase \textit{Drosophila} lamins Dm\textsubscript{1} and Dm\textsubscript{2} are highly phosphorylated (2-3 phosphates per molecule). The function of this phosphorylation is unknown. Using a procedure designed to

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*Mutants with reduced binding to ADL84 as determined by immunoblotting were sequenced throughout the region coding for the head domain (lamin amino acids 1-56). Three mutants (R\textsuperscript{22} W, S\textsuperscript{25} P, and P\textsuperscript{28} L) were sequenced throughout their coding region and found not to contain any additional mutations.

Amino acid sequence of \textit{Drosophila} lamin Dm\textsubscript{0} head; the probable ADL84 epitope is shown in bold: M\textsuperscript{1}SSKSSRAGTLATPQPGNTSTPR\textsuperscript{2}PPSAGPQP\textsuperscript{P}PPPSTHSQTA\textsuperscript{S}SSLSPTRHS\textsuperscript{R}VRAEKV\textsuperscript{56}.  

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**Fig. 5.** Epitope mapping of ADL84; screening for point mutants which abolish binding to ADL84. Random mutations were generated in the coding sequence of DmL-179. The plasmid was transformed into \textit{E. coli} strain HMS174(DE3)pLysS. Resulting colonies were transferred to nitrocellulose, protein expression was induced with IPTG and colonies were lysed by freezing and thawing. Filters were double-labeled with ADL84 (using alkaline phosphatase-conjugated anti-mouse IgG secondary antibodies and colorimetric detection; A) and polyclonal affinity-purified rabbit anti-lamin IgG (using peroxidase-conjugated anti-rabbit secondary antibodies and enhanced chemiluminescence; B). Arrows indicate colonies producing mutated DmL-179 that do not bind ADL84.

**Fig. 6.** Epitope mapping of ADL84; analysis of selected mutants by immunoblotting. Selected colonies (as shown in Fig. 5) were grown in liquid culture, induced with IPTG, lysed by boiling in SDS-containing sample loading buffer and separated on SDS-10\% polyacrylamide gels. Duplicate gels were transferred to nitrocellulose and probed with ADL84 (A) or with polyclonal affinity-purified rabbit anti-	extit{Drosophila} lamin IgG (B). Lanes 1,2, selected mutants; lane WT, colony containing the unmodified plasmid coding for DmL-179.
A peptide consisting of amino acids 18-32 of the lamin Dm0 (Fig. 6 and Table 1). We confirmed this by showing that a middle of the epitope is a single serine residue, located at sequence inhibits binding of ADL84 to lamin Dm0. In the epitope for ADL84 consists of the sequence R22PPSAGP (Fig. 6 and Table 1). We confirmed this by showing that a position 25. Phosphorylation of this serine reduced the ability of the peptide to inhibit binding of ADL84 to lamin Dm0 by about 19-fold. The residual inhibition might be caused by incomplete chemical phosphorylation of the peptide, contaminating phosphatase activity during the assay, or it might reflect a reduced, but measurable affinity of the phosphorylated peptide for ADL84. These data indicate that phosphorylation of serine 25 distinguishes lamin Dm2 from lamin Dm1.

The methodology used to define the ADL84-epitope (random mutagenesis followed by screening with both monoclonal and polyclonal antibodies) was both rapid and efficient. A similar approach was used by Ikeda et al. (1992) to map epitopes of two anti-E. coli recA protein mAbs. We modified their method in a number of ways. By substituting a T7 RNA polymerase-dependent expression system for λgt11, we were able to both express and sequence mutated proteins without the need for subcloning. Use of an E. coli strain expressing the gene for bacteriophage T7 lysozyme facilitated antibody screening in that colonies could be efficiently lysed simply by freezing and thawing. The utility of this approach for antibody screening of expressed protein fragments was demonstrated in this article. This approach may also prove useful for high-resolution mapping of polypeptide domains involved in specific protein-protein interactions.

In conclusion, the most immediate question regarding the conversion of lamin Dm1 to lamin Dm2 concerns its physiological/functional significance. The highly dynamic nature of the event as well as the apparently quantitative conversion of lamin Dm2 to lamin Dm1 during heat shock (Smith et al., 1987) suggests that it may be involved in such processes as growth of the nuclear envelope during interphase and/or regulation of gene expression. The identification of the site involved in the posttranslational conversion (via phosphorylation) of lamin Dm1 to lamin Dm2 will permit us to follow the conversion in vivo and to explore the functional consequences of this conversion. The authors thank Mike Frohman (Stony Brook, NY) for the stimulating discussions concerning the use of PCR-mediated random mutagenesis, Sergei Bogachev (Novosibirsk, Russia) for performing
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